REMARKS

Claims 1, 3-16, and 18-39 are currently pending in the application. Claim 1 has been amended to incorporate the limitation of claim 3 and to remove the possibility of having 2'-substituted ribonucleotides only at the 3'-terminal end of the nucleotide. Claim 16 has been amended to be directed to a method for introducing an intact oligonucleotide into a mammal, support for which is found, *inter alia*, in original claims 37-39. Claim 4 has been amended herein to correct a dependency and to correct an obvious typographical error. Claim 15 has been amended to indicate that the antiviral activity is against HIV-1 or HIV-2, support for which is found, *inter alia*, in original claim 14. Claims 3, 12, 13, 27, and 28 have been cancelled herein without prejudice or disclaimer of the subject matter claimed therein. Accordingly, no new matter is introduced by these amendments. Accordingly, after entry of these amendments, claims 1, 4-11, 14-16, 18-26, and 29-39 will be pending in the application.

The outstanding rejections are addressed individually below.

1. *Claims 16, 18-30 and 34-38 are enabled.*

Claims 16, 18-30, and 34-38 stand rejected under 35 U.S.C. § 112, first paragraph, for allegedly lacking enablement over the scope claimed. Applicant respectfully traverses this rejection.

Claims 27 and 28 have been cancelled herein. Accordingly, Applicant respectfully submits that the rejection is moot with respect to these claims.

M.P.E.P § 2164.01 states that 35 U.S.C. § 112, first paragraph, "has been interpreted to require that the claimed invention be enabled so that any person skilled in the art can make and use the invention without undue experimentation." The same section further states that "[t]he fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation."

The specification does teach one of skill in the art to how to *make* the invention (*see, e.g.*, the specification at page 13, line 26 to page 15, line 30 and Example 1, page 32, line 13 to page 33, line 2).

Additionally, the specification does teach one of skill in the art how to *use* the invention (*see, e.g.*, the specification at page 20, line 10 to page 21, line 30 (describing pharmaceutical formulations), page 21, line 32 to page 24, line 8 (describing therapeutically acceptable methods and amounts), page 24, lines 10-20 (describing methods of administration), and page 24, line 22 to page 26, line 24 (describing therapeutic formulations and pharmaceutical compositions)).

Therefore, the specification has fully enabled the invention as claimed because it teaches how to make and use the invention without undue experimentation.

Furthermore, the specification provides examples indicating that the invention *does* work as claimed. To start with, the oligonucleotides of the invention have been tested *in vitro* in a variety of cell types. The specification indicates that *in vitro* experiments were performed analyzing the ability of the oligonucleotides of the invention to inhibit existing infections and to protect against infection in MT-4 cells (page 19, lines 18-25) The results of there experiments are shown in FIGS: 1 and 2. Additional experiments were performed analyzing the preclinical range of anti-HIV activity of various oligonucleotides of the invention (page 26, line 26 to page 30, line 8). Other experiments analyzed toxicity in fresh human peripheral blood mononuclear cells (page 30, lines 10-28). Several of these experiments are also detailed in the Examples 7 and 8 (page 42, line 13 to page 47, line 27), which show anti-HIV activity in fresh human peripheral blood lymphocytes and fresh human monocyte-macrophages as well as inhibition of acute infection of MT-4 cells.

Furthermore, the specification at page 31, line 23 to page 32, line 5, and Example 9 (page 47, line 29 to page 52, line 23) of the instant patent application provides description of *in vivo* testing experiments to evaluate the presence of oligonucleotides of the invention in tissue after oral administration *in vivo*. The specification at page 30,

line 30 to page 31, line 7 indicates the bioavailability of oligonucleotides of the invention in rats and monkeys *in vivo*, and the specification at page 31, lines 9-21 indicates the absorption of oligonucleotides of the invention in both rats and monkeys *in vivo*.

More specifically, the specification as amended states at page 30, line 30 to page 31, line 4 that "the bioavailability of Oligo 12 was examined *in vivo* and was found to be intravenously and orally bioavailable to rats and monkeys after a single dose. . . . [and] synthetic oligonucleotides systemically administered to pregnant murine females were found to cross the placenta and be available in the blood of embryos *in utero*." Furthermore, the specification at page 31, lines 14-21 states that an oligonucleotide of the invention "was found to be absorbed through the gatstrointestinal tract and accumulated in various organs and tissues" following intravenous or oral administration.

Therefore, the specification provides information indicating that the claimed invention does work *in vivo* in accepted animal models.

Furthermore, claims 16, 18-26, 29-30, and 37-39 (as amended) do not require that the oligonucleotide inhibit the proliferation of HIV-1 or HIV-2. These claims are directed to methods for introducing an intact oligonucleotide into a mammal, and merely require that the oligonucleotide is present in intact form in the systemic plasma following administration. The specification provides support for these claims at, *inter alia*, page 30, line 30 to page 31, line 4, where it is disclosed that Oligo 12 was found to be intravenously and orally bioavailable to rats and monkeys after a single dose, which is indicative of the presence of the intact oligonucleotide.

Additionally, claims 34-36 are directed to a method of inhibiting HIV-1 or HIV-2 infection in a cell. As discussed above, Examples 7 and 8 (page 42, line 13 to page 47, line 27) show anti-HIV activity in fresh human peripheral blood lymphocytes and fresh human monocyte-macrophages as well as inhibition of acute infection of MT-4 cells.

The Office Action states that "neither the in vitro demonstration of anti-HIV activity, nor the oral bioavailability of oligonucleotides in an organism provide the

enablement for the ability to treat and/or inhibit HIV-1 or 2 infection in an organism. In vitro results cannot be extrapolated to in vivo efficacy." As described above, amended claims 16, 18-26, 29-30, and 37-39 (as amended) do not require that the oligonucleotide inhibit the proliferation of HIV-1 or HIV-2.

However, to the extent that this statement is applicable to claims 34-36, Applicants submit that M.P.E.P § 2164.02 states that

> [a]n *in vitro* or *in vivo* animal model example in the specification, in effect, constitutes a "working example" if that example "correlates" with a disclosed or claimed method invention. . . . In this regard, the issue of "correlation" is also dependent on the state of the prior art. In other words, if the art is such that a particular model is recognized as correlating to a specific condition, then it should be accepted as correlating unless the examiner has evidence that the model does not correlate.

This section further states that a "rigorous or an invariable exact correlation is not required . . . ." M.P.E.P § 2164.03 relates to the relationship of predictability of the art and the enablement requirement; this section states that "what is known in the art provides evidence as to the question of predictability."

In the Amendment Under 37 C.F.R. § 1.111, filed January 28, 2003, Applicants discussed several published articles regarding the state of antisense technology and corroborating that antisense oligonucleotides have been shown to be effective. Thus, Applicant respectfully asserts that the Examiner's concerns regarding the correlation between *in vitro* and *in vivo* use are unfounded and inappropriate.

Accordingly, Applicant respectfully requests that this rejection under 35 U.S.C. § 112, first paragraph, be reconsidered and withdrawn.

2. ***Claims 1, 3-15, and 31-36 are not obvious over Agrawal et al. in view of Goodchild et al. and Hovanessian et al.***

Claims 1, 3-15, and 31-36 stand rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Agrawal *et al.*, in view of Goodchild *et al.* and Hovanessian *et al.* Applicant respectfully traverses this rejection.

Claims 3, 12, and 13 have been cancelled herein. Accordingly, Applicant respectfully submits that this rejection has been rendered moot with regard to these claims.

The subject claims relate to a synthetic oligonucleotide having a nucleotide sequence specifically complementary to nucleotides 324 to 345 of a conserved *gag* region of the HIV-1 genome set forth as SEQ ID NO: 5, the oligonucleotide consisting of 21 nucleotides which are linked via phosphorothioate internucleotide linkages, wherein the oligonucleotide comprises at least two 5′ terminal ribonucleotides, or at least two 3′-terminal and at least two 5′ terminal ribonucleotides, and wherein the ribonucleotides are 2′-substituted ribonucleotides. Also covered in these claims are a pharmaceutical formulations and methods of inhibiting HIV-1 or HIV-2 infection in a cell.

M.P.E.P. § 716.01(a) states that

> [t]he Court of Appeals for the Federal Circuit stated in *Stratoflex, Inc. v. Aeroquip Corp.*, . . . that "evidence rising out of the so-called 'secondary considerations' must always when present be considered en route to a determination of obviousness." Such evidence might give light to circumstances surrounding the origin of the subject matter sought to be patented. As indicia of obviousness or unobviousness, such evidence may have relevancy.

(citations omitted) M.P.E.P. § 716.02 further states that "[a]ny differences between the claimed invention and the prior art may be expected to result in some differences in properties. The issue is whether the properties differ to such an extent that the difference is really unexpected." As stated in M.P.E.P. § 716.02(a), "[e]vidence of unobvious or unexpected advantageous properties, such as superiority in a property the claimed compound shares with the prior art, can rebut *prima facie* obviousness" and "[a]bsence of property which a claimed invention would have been expected to possess based on the teachings of the prior art is evidence of unobviousness." (M.P.E. P. 700-240)

Work published after the filing of the priority application in this case indicates that the oligonucleotides of the present invention produced unexpected results. As discussed in Agrawal (*Biochimica et Biophysica Acta* 1489:53-68 (1999), attached hereto as Attachment A), the presence of a CpG motif in phosphorothioate oligonucleotides adds to the severity of the toxicity of the oligonucleotide. (Agrawal, page 58) The sequence to which the claimed oligonucleotides are complementary, nucleotides 324 to 345 of a conserved *gag* region of the HIV-1 genome, contains one CpG motif. However, this paper teaches that incorporation of 2'-O-methylribonucleosides at both the 3'- and 5'-ends of an oligonucleotide complementary to the *gag* gene of HIV-1, in which the CpG motif was also modified, produced an oligonucleotide which showed reduced toxicity. (Agrawal, page 60) Furthermore, incorporation of 2'-O-methylribonucleosides at both the 3'- and 5'-ends of an oligonucleotide complementary to the *gag* gene of HIV-1, in which the CpG motif was not modified, produced an increase in toxicity. (Agrawal, page 60)

As a result of the positioning of the nucleotide sequence to which the specific 21mers of the present invention are complementary, any modification to at least two nucleotides at the 5' end of the oligonucleotide, such as having 2'-substituted ribonucleotides at the 5' end, will modify at least the C of the CpG sequence. Thus, as a result of the specific sequence to which the antisense oligonucleotide is complementary and the modifications thereto, the oligonucleotides of the present invention have decreased toxicity, which is an unexpected result, given the presence of a CpG motif in the nucleotide sequence.

The '721 patent does not teach or suggest any motivation for selecting the particular 21mer nucleotide sequence of the claimed invention.

Furthermore, one of skill in the art would not be motivated to combine the primary reference (the '721 patent) with Goodchild *et al.* or Hovanessian *et al.* to achieve the claimed invention.

Goodchild *et al.* refers to the use of the initiator codon for the *gag* gene as a possible sequence to which an oligonucleotide could be complementary, but does not refer to the nucleotides 324 to 345 of the *gag* gene, itself, and does not suggest using the *gag* gene, itself, as such a sequence.

Hovanessian *et al.* relates to transmembrane envelope proteins of HIV-2. Hovanessian *et al.* does not teach or suggest antisense oligonucleotides. Furthermore, Applicant submits that this reference teaches away from the expectation that the *in vitro* inhibition of *gag* expression by antisense would lead to the *in vitro* inhibition of HIV-1 and HIV-2 replication *in vitro*, by emphasizing the differences between HIV-1 and HIV-2, rather than their similarities. For example, this patent states that HIV-2 and SIV-mac share about 75% overall nucleotide sequence homology, but both of them are only distantly related to HIV-1 with about 40% overall homology (col. 1, lines 31-34).

Therefore, one of skill in the art would not be motivated to combine these references with the '721 patent.

Accordingly, Applicant respectfully requests that the obviousness rejection of claims 1, 3-15, and 31-36 under 35 U.S.C. § 103(a) be reconsidered and withdrawn.

CONCLUSIONS

In view of the arguments set forth above, Applicant respectfully requests reconsideration and reexamination of the above-referenced patent application. Applicant submits that the rejections contained in the Office Action mailed on April 22, 2003, have been overcome, and that the claims are in condition for allowance.

Applicant encloses herewith a Petition for a Two Month Extension of Time pursuant to 37 C.F.R. § 1.136, until September 22, 2003, to respond to the Examiner's Office Action mailed on April 22, 2003. Please charge our Deposit Account No. 08-0219 the $205.00 fee for this purpose.

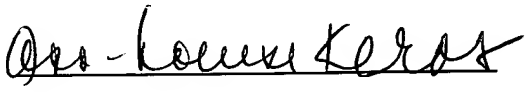
Applicant also herein requests continued examination of the application according to 37 C.F.R. § 1.114. Please charge the same deposit account the $375.00 fee set forth in 37 C.F.R. § 1.17(e) for this purpose.

Applicant also encloses herewith an Information Disclosure Statement. As this submission is being filed concurrently with a Request for Continued Examination, no additional fees are believed to be due in connection with this submission in accordance with 37 C.F.R. § 1.97(b)(4).

No other fees are believed to be due in connection with this response. However, please charge any underpayments or credit any overpayments to Deposit Account No. 08-0219.

If the Examiner believes that any further discussion of this communication would be helpful, please contact the undersigned at the telephone number provided below.

Respectfully submitted,



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Attachments: Reference cited in response (Agrawal, *Biochimica et Biophysica Acta* 1489:53-68 (1999))

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Review

Importance of nucleotide sequence and chemical modifications of antisense oligonucleotides

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Abstract

The antisense approach is conceptually simple and elegant; to design an inhibitor of a specific mRNA, one needs only to know the sequence of the targeted mRNA and an appropriately modified complementary oligonucleotide. Of the many analogs of oligodeoxynucleotides explored as antisense agents, phosphorothioate analogs have been studied the most extensively. The use of phosphorothioate oligodeoxynucleotides as antisense agents in various studies have shown promising results. However, they have also indicated that quite often, biological effects observed could be solely or partly non-specific in nature. It is becoming clear that not all phosphorothioate oligodeoxynucleotides of varying length and base composition are the same, and important consideration should be given to maintain antisense mechanisms while identifying effective antisense oligonucleotides. In this review, I have summarized the progress made in my laboratory in understanding the specificity and mechanism of actions of phosphorothioate oligonucleotides and the rationale for designing second-generation mixed-backbone oligonucleotides.

Keywords: Antisense; Oligonucleotide; Mixed-backbone oligonucleotide; Pharmacokinetics; Immune stimulation; CpG; Pro-drug; Antiviral

Contents

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1. Introduction

Antisense oligonucleotides provide a rationally designed tool to manipulate expression of specific gene products [1,2]. In the last 10 years, antisense oligonucleotides have been widely used in various in vitro and in vivo models [3–11]. Based on the promising results obtained, antisense oligonucleotides are being explored for their potential as therapeutic agents for the treatment of viral infections, cancers and inflammatory disorders [5,6,8,9]. The antisense approach is conceptually simple; to design an inhibitor, one needs only to know the sequence of the targeted gene product (mRNA) and to identify any specific modifications of the oligonucleotide. Antisense oligonucleotides have been used extensively in the last several years, with mixed results. [8,10–12]. The most-studied of the oligodeoxynucleotides are the phosphorothioate analogs (PS-oligos), in which one of the non-bridging oxygen atoms in the phosphate backbone is replaced by a sulfur [3–11]. Based on the results obtained to date with PS-oligos, it has become evident that PS-oligos exert biological activity by multiple mechanisms of actions [8–13]. The mechanisms of action can be classified into three categories: (a) sequence-specific activity by binding to mRNA, referred here as antisense activity; (b) sequence-specific activity by interacting with other factors than mRNA, referred here as non-antisense activity; and (c) non-sequence-specific activity. In this review, I have attempted to summarize the progress in my laboratory in understanding the rules that govern the specificity and mechanisms of actions of PS-oligos and the rationale for designing second-generation antisense oligonucleotides. A number of other investigators have published their results and views, which also appear in this issue.

2. Phosphorothioate oligodeoxynucleotides

PS-oligos have a negatively charged backbone and are capable of supporting RNase-H activity similar to phosphodiester oligodeoxynucleotides, but PS-oligos have greater resistance to nuclease degradation than do phosphodiesters. These intrinsic properties have made PS-oligos the choice as first-generation antisense oligonucleotides [3–11].

We and others initially used PS-oligos as inhibitors of HIV-1 replication in HIV-1-infected cells [14–23]. From the results obtained it was obvious that the PS-oligos effectively inhibited HIV-1 replication, but the apparent mechanism of HIV-1 inhibition differed depending on the experimental model [14–22]. PS-Oligos inhibited HIV-1 replication by antisense mechanisms as well as non-sequence-specific mechanisms [14–25]. The non-sequence-specific mechanism was most probably due to the polyanionic nature of PS-

oligos [14–25]. Similar results were observed with PS-oligos used to inhibit replication of influenza virus and other viruses [26]. In some recent studies, the biological activity observed with PS-oligos has also been associated with their polyanionic nature [27,28].

In the last six years, there have been many reports in which PS-oligos have been used to inhibit overexpression of cellular gene products implicated in cancer and inflammation. The results strongly suggest that the inhibition observed in these studies was primarily due to antisense activity [3–11,30–36]. We recently used PS-oligos complementary to an MDM2 oncogene [37]. The MDM2 oncogene encodes for an inhibitor of the p53 tumor suppressor protein that regulates p53 in a negative feedback loop [38,39]. In this study, selected antisense PS-oligos inhibited MDM2 expression at both the mRNA and protein levels [37]. Suppression of MDM2 oncoprotein led to a decrease in MDM2-p53 complex formation, which in turn resulted in an increase in p53 transcriptional activity, and finally to apoptosis [37]. The effects observed were sequence specific, as the control PS-oligos (with four mismatches) did not show such activity.

It has been shown in a number of studies that PS-oligos with CpG motifs have immune-stimulatory properties in rodents [11,40–46]. The severity of immune stimulation depends on the position of the CpG motif and its flanking sequence of PS-oligos [40–46]. PS-Oligos containing CpG motifs are known to induce cytokines, including IL-6, IL-12, TNF-α, gamma-IFN [43,44], and also chemokines [45]. These cytokines induced by PS-oligos containing CpG motifs have been shown directly or indirectly to have antiviral [47,48], anticancer [49], and antibacterial activities [50].

3. Pharmacokinetics and tissue distribution

Pharmacokinetics of PS-oligos in mice following intravenous administration showed rapid elimination from the plasma compartment with half-lives ranging from 30 min to 1 h [51]. PS-Oligo was distributed to highly perfused organs, such as kidney, liver, bone marrow, and spleen, in higher concentration than other tissues [51]. The primary route of elimination was in urine, with smaller amounts found in feces [51]. Following intraperitoneal or subcutaneous administration, no significant differences in tissue distribution were observed, except that lower maximum plasma concentrations were achieved than with intravenous administration [51,52]. The analysis of extracted PS-oligos from plasma and tissues showed the presence of both intact and degraded forms of the PS-oligo [51,52]. Protection of PS-oligos on the 3′-end significantly minimized degradation, this suggested that in vivo degradation was primarily due to 3′-exo-nucleases [53]. Detailed analysis of the extracted oligo showed that the PS-oligo was degraded primarily from the 3′-end, but some degradation products were generated following degradation from the 5′-end and from both the 3′- and 5′-ends [54]. Similar pharmacokinetic and tissue disposition results were obtained in rats [55] and monkeys [56]. In humans, the plasma pharmacokinetic profile and elimination in urine were similar to those observed in monkeys [57]. Similar results, in general, have been reported with PS-oligos of varying sequences [58]. The pharmacokinetics of PS-oligos are found to be largely sequence independent except for PS-oligos that can form hyperstructures (e.g., G-rich oligos) [59].

4. Safety

Safety studies of PS-oligos in mice and rats show sequence-dependent side effects [10,11]. These side effects include splenomegaly, thrombocytopenia, and elevation of the liver enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) [10,11,60]. Histopathological examinations have revealed multi-organ mononuclear cells infiltrates, reticuloendothelial cell and lymphoid hyperplasia, and renal tubule degeneration [60]. The severity of these side effects are dependent on the dose and the frequency and duration of administration [60]. Similar results have been reported with other PS-oligos [61].

The safety profile of PS-oligos in monkeys is different from that observed in mice and rats [62]. Intravenous administration of PS-oligos in monkeys caused a brief increase, followed by a prolonged decrease, in arterial blood pressure, and a transient decrease in peripheral total white blood cells and

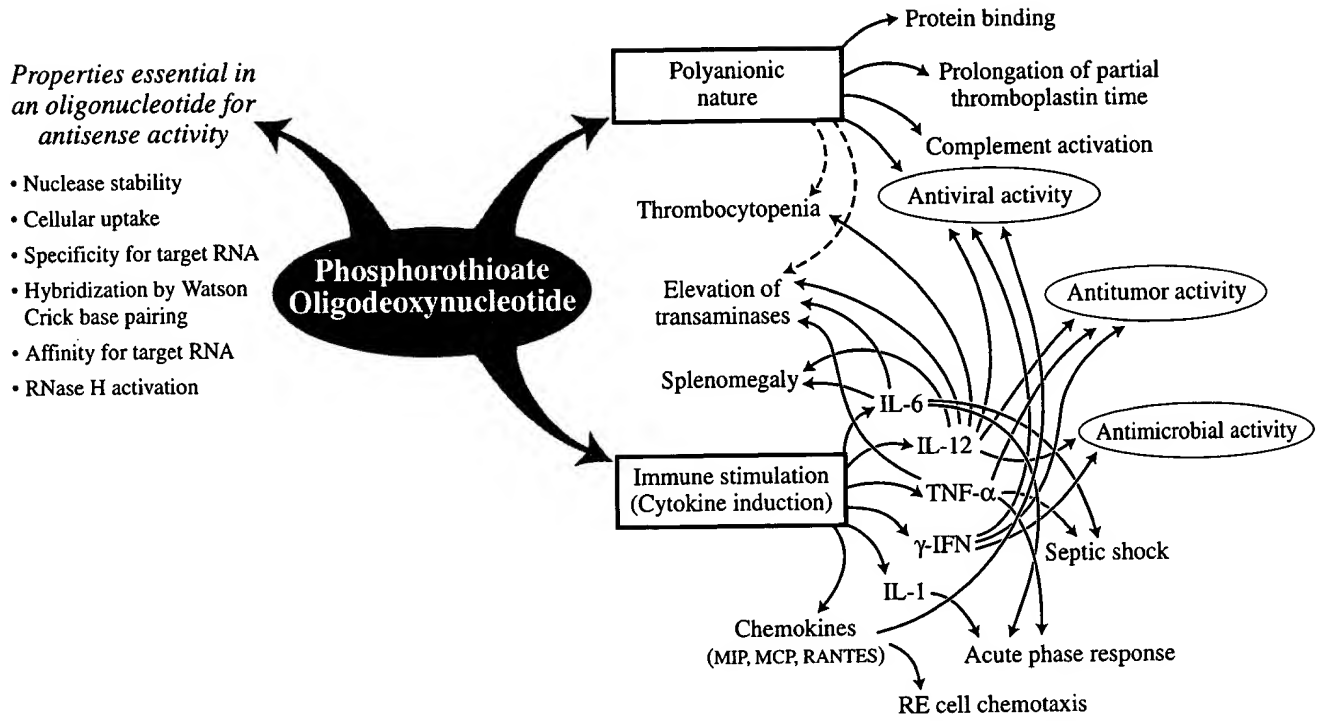

Fig. 1. Factors affecting the mechanism of action of oligonucleotides. Antisense oligonucleotides are designed to bind to targeted mRNA and inhibit translation. An effective oligonucleotide should be stable to nucleases and taken up by cells. In addition, oligonucleotides should have strong affinity for the target mRNA and should activate RNase H for RNA cleavage. Based on the nucleotide sequence and the nature of the internucleotide linkages, however, oligonucleotides display additional properties that may interfere with the specificity and mechanism of action of the oligonucleotide. Two major properties are the polyanionic nature and immune stimulation. As shown above in a generalized way, the polyanionic nature and immune stimulation may produce biological activities and side effects that compromise the specificity and action of a given oligonucleotide. Minimization of polyanionic nature and immune stimulatory properties by appropriate chemical modification should be considered in second-generation oligonucleotides.

neutrophil counts [62]. In addition, complement activation and prolongation of aPTT have also been observed [62]. These side effects are sequence independent, however, dependent on the concentration of PS-oligo in the plasma compartment, and can be minimized by slow intravenous infusion [62]. A similar observation has been made with other PS-oligos [63]. PS-Oligos that can form hyperstructures (e.g., G-rich oligos) have greater effects on complement activation and prolongation of aPTT [64].

A PS-oligo (GEM 91) has been administered to humans by 2- or 24-h intravenous infusion. The side effects observed included thrombocytopenia, elevation of transaminases, and prolongation of aPPT [65]. These side effects were dependent on the dose and duration of the treatment and the frequency of administration. Administration of lower doses of PS-oligos had minimal changes in the above mentioned side effects [65–67].

5. Understanding of PS-oligos

On the basis of knowledge gained to date with PS-oligos, the following generalizations can be made.

1. PS-oligos have built-in properties (e.g., affinity for target mRNA, nuclease stability, cellular uptake, and RNase H activation) required for antisense activity (Fig. 1).
2. PS-oligos behave like polyanionic molecules as is evident from the inhibitory activity observed in viral replication assays, binding to growth factors, serum proteins, etc. In addition, activation of complement and prolongation of aPTT in monkeys and humans is associated with the polyanionic characteristics of PS-oligos (Fig. 1).
3. PS-oligos have sequence-specific biological activities, which are not due to hybridization to specific mRNA. Two types of mechanisms may be in-

volved: immune stimulation (Fig. 1) and binding of factors (e.g., transcription factor and proteins) to a decoy or specific motif of PS-oligos [67–69].

6. Beyond PS-oligos

The use of PS-oligos in various studies has shown that antisense oligonucleotides can be used to selectively inhibit the expression of targeted mRNA in cell culture and in vivo [3–11,29–36]. Certain undesirable properties (e.g., polyanionic nature, immune stimulation) limit their potential for wider use as therapeutic agents and also as tools in elucidating the function of specific target genes (Fig. 1).

To overcome some of the limitations of PS-oligos, extensive efforts have been made to synthesize various analogs of oligonucleotides [3–11,70]. These analogs include deoxynucleotides with modification of the internucleotide linkages, heterocyclic bases, or sugar [3–11,70]. Some of these analogs have higher affinity for target RNA and increased resistance to serum and cellular nucleases than PS-oligos. Studies of these analogs in various cell culture models have not yielded encouraging results compared with those obtained with PS-oligos.

In the last few years, we have made attempts to rationally minimize the undesirable properties of PS-oligos, while maintaining the properties essential for antisense activity (Fig. 1). The following are two examples in this respect.

7. Minimization of polyanionic nature of PS-oligos

The polyanionic characteristics of PS-oligos have been associated with some of the observed non-sequence-specific biological activities [14,15,21,23,25–28] and also with complement activation and prolongation of aPTT [62,71,72]. In our studies using phosphorothioate oligoribonucleotides, phosphorothioate 2′-*O*-methyloligoribonucleotides, and phosphorothioate 2′,5′-oligoribonucleotides, it became clear that the polyanionic nature of these analogs is somewhat different from that observed with PS-oligos [73]. These analogs had less of an effect on complement activation and prolongation of aPTT, suggesting that not only the phosphorothioate linkage, but also the nature of the nucleosides of PS-oligos, is responsible for the polyanionic characteristics. It is possible that Rp and Sp diastereomers of PS-oligos have different polyanionic characteristics; one of the isomers of PS-oligoribonucleotides has less pronounced polyanionic characteristics because of the 2′-hydroxyl group or other 2′-modifications.

There have been no reports on the polyanionic characteristics of Rp and Sp stereospecific PS-oligos. We have observed that Rp stereospecific PS-oligos have a greater affinity for complementary RNA and less stability towards nucleases than do stereo-random PS-oligos [74]. Also, Rp PS-oligos were better substrates for RNase H than were stereo-random PS-oligos [74]. Independent studies using Sp stereospecific PS-oligos have shown that they have less of an affinity for complementary RNA and greater stability towards nucleases than do Rp and stereo-random PS-oligos [75]. Lack of efficient synthetic methodologies to obtain stereospecific Rp or Sp PS-oligos have been preventing us from performing detailed studies. Recently, however, we have succeeded in synthesizing stereo-enriched Rp and Sp PS-oligos and also PS-oligos with an appropriate mix of Rp and Sp linkages [76,77]; detailed studies are now under way.

To minimize the polyanionic nature of the PS-oligos, we have taken advantage of the reduced polyanionic characteristics of 2′-*O*-methylribonucleosides, (as observed by reduced complement activation and prolongation of aPTT), and have substituted a few deoxynucleosides with 2′-*O*-methylribonucleosides, either at the 3′-end or both the 3′- and 5′-ends or in the center of the PS-oligos [78–80] (Fig. 2). The overall result of this substitution is increased affinity to target RNA, stability towards nucleases, and reduced polyanionic-related side effects. In addition, these oligonucleotides retain the RNase H activating capability due to the presence of PS-oligo [78–80]. Similar results have been obtained by incorporating other modified oligonucleotides [81–83]. In addition to chemical modifications, polyanionic related effects can be minimized with the use of appropriate formulations. Use of protamine has been shown to minimize these effects [72].

Table 1
Structure and sequence of oligonucleotides

1.	TCG TCG CTG TCT CCG CTT CTT CTT GCC
2.	^{me}TCG ^{me}TCG CTG TCT C^{me}CG CTT CTT CTT GCC
3.	TC$_{m}$G TC$_{m}$G CTG TCT CC$_{m}$G CTT CTT CTT GCC
4.	[TCG] [TCG] CTG TCT C[CG] CTT CTT CTT GCC
5.	CTC TCG CAC CCA TCT CTC TCC TTC T
6.	CTC TC$_{m}$G CAC CCA TCT CTC TCC TTC T
7.	CTC TGC CAC CCA TCT CTC TCC TTC T
8.	CTC ^{me}TCG CAC CCA TCT CTC TCC TTC T
9.	[CG C]AC CCA TCT CTC TCC [UUC U]
10.	GCG TGC CTC CTC ACT GGC
11.	CGC CGG GAT CTC GAT GCT CAT
12.	CCG CTC TTC CTC ACT GGT
13.	[GCG U]GC CTC CTC AC[U GGC]
14.	CTC TCG CAC C$_{m}$C$_{m}$A T$_{m}$C$_{m}$T C$_{m}$T$_{m}$C TCC TTC T
15.	CTC TCG CA[C CCA UCU C]TC TCC TTC T
16.	CTC TCG CA[C CCA UCU C]TC TCC TTC T

All sequences are phosphorothioate; ^{me}C - 5-methyl cytosine; X$_{m}$X - methylphosphonate linkage; [XX] - 2'-O-methyl ribonucleoside; [XX] - 2'-O-methylribonucleoside with phosphodiester linkages.

Oligo **1** is complementary to *rev* gene of HIV-1; oligo **5** is complementary to *gag* gene of HIV-1; oligo **10** is complementary to R1α subunit of human protein kinase A (PKA); oligo **11** is complementary to RIIβ subunit of human PKA; oligo **12** is complementary to R1α subunit of mouse PKA.

8. Minimization of immune stimulation by PS-oligos

PS-oligos containing a CpG motif and the appropriate flanking sequences are known to be immune stimulatory [11,40–46]. Recent studies have indicated that activation of immune stimulation is through induction of mitogen-activated protein kinases [84,85]. The immune stimulation by CpG-containing PS-oligos results in induction of various cytokines, which has a therapeutic effect; these PS-oligos are being developed as novel therapeutic agents [47–50]. At the same time, these cytokines at higher doses have been associated to side effects [45].

We have studied PS-oligos for their safety profile in mice and rats and have reached the conclusion that the presence of CpG motif in PS-oligos adds to the severity of the toxicity observed [11]. For example, oligo **1** (Table 1) administered to mice causes thrombocytopenia, elevation of transaminases, and enlargement of the spleen (Fig. 3A). In addition, histopathological changes in the kidney, liver, and spleen were also noted. Modification of the CpG motif significantly minimized the side effects observed with oligo **1**; these modifications included: replacement of the cytosine of the CpG motif with a 5′-methyl cytosine (oligo **2**, Table 1); replacement of the phosphorothioate linkage of the CpG motif with a methylphosphonate linkage (oligo **3**, Table 1); and replacement of the entire CpG motif with 2′-*O*-methylribonucleosides (oligo 4, Table 1) (Fig. 3A). Minimization of the histopathological changes was also noted with oligos **2**, **3**, and **4**. These modifications suppressed the immune stimulatory properties of the CpG motif [42]. Reduction in the toxicity of oligo **1** produced by these modifications strongly suggests that PS-oligos have similar toxicities, but the severity of toxicity is increased due to presence of CpG motif [11] and their immune-stimulatory properties. Similar results were observed in rats with oligos **5**, **6**, **7**, and **8** (Fig. 3B).

It is important to note that the flanking sequence of the CpG motif is a major factor in inducing immune stimulation, and not all PS-oligos with the CpG motif will behave in the same manner [11,41]. Oligos **10**, **11**, and **12**, which contain the CpG motif at different positions in their sequences and flanking sequences, had significantly different toxicity profiles in mice (Fig. 3C). Oligo **10** showed more toxicity than oligo **11** and **12**. It is also important to note that immune stimulation due to a given PS-oligo sequence depends on the host, the dose, and the route of administration (Q. Zhao, S. Agrawal, unpublished data).

The above discussion and results suggest that a given PS-oligo can be appropriately modified to sup-

press its immune-stimulatory properties and the resulting toxicity. Furthermore, these modifications can be made more rationally to also improve general therapeutic potential of oligonucleotides as discussed below.

9. Mixed-backbone oligonucleotides (MBOs)

As it is evident from the above discussion, the nucleotide composition and nature of the nucleotide and internucleotide linkages alone or in combination dictate the biophysical, biochemical, and biological properties of oligonucleotides [70]. A number of oligonucleotides analogs have been studied that display properties different from those of PS-oligos in terms of resistance to nucleases, affinity to target RNA, cellular uptake, activation of RNase H, and more importantly, the in vivo pharmacokinetic profile [3–11]. In our earlier studies, we employed phosphorothioate oligoribonucleotides which bind to RNA with higher affinity than PS-oligos, but do not activate RNase H. They showed reduced anti-HIV activity compared to PS-oligos [17]. These results suggested that for optimum activity, antisense oligonucleotides should have combination of various properties instead of only increased stability toward nucleases or high affinity to target RNA.

We have made attempts to combine two modified oligonucleotides in order to generate a mixed-backbone oligonucleotide (MBO) that brings together the beneficial properties of the two molecules [71,72,78,80–82,86,87]. MBOs in general have two segments: one that contains an oligonucleotide analog capable of activating RNase H, and another that does not activate RNase H (Fig. 2). The oligonucleotides that activate RNase H are those bearing at least four contiguous phosphodiester or phosphorothioate

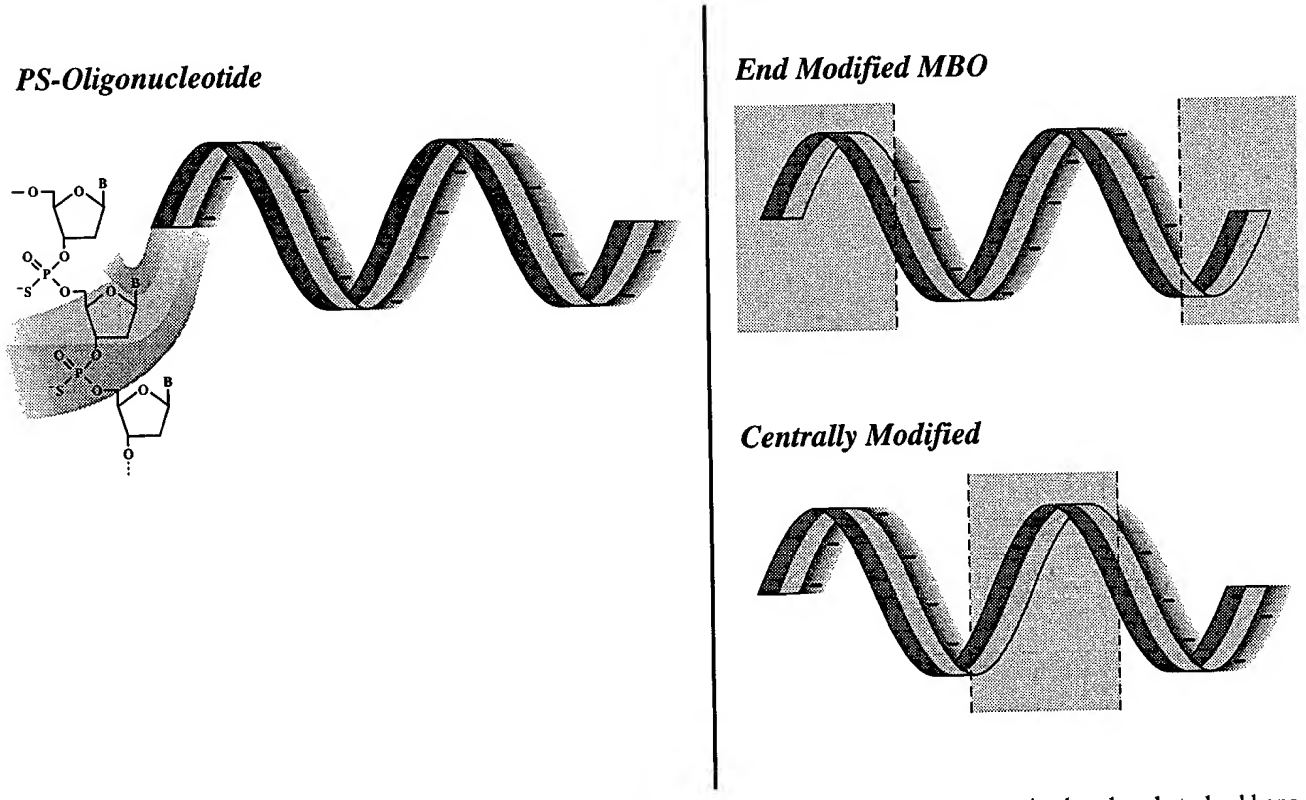

Fig. 2. Structure of PS-oligonucleotide and MBOs. In PS-oligos one of the non-bridging oxygen atoms in the phosphate backbone is substituted by a sulfur. In general, various biophysical and biochemical properties of PS-oligos are controlled by phosphate backbone, nucleoside sugar, and heterocyclic bases. To modulate many of these properties, mixed-backbone oligonucleotides have been studied. MBOs refer to oligonucleotides which combine the advantages of two different modified oligonucleotides. Based on the position of incorporation of modified oligonucleotides in PS-oligos, MBOs can be classified as end-modified MBOs and centrally modified MBOs.

internucleotide linkages [80,81]. Of the oligonucleotide analogs that do not activate RNase H, we have studied oligodeoxynucleotides containing methylphosphonate [81–83,86,87], phosphoramidate [81,88], methylthiophosphonate [89], methylphosphotriester [90], methylphosphothiotriester [90], carbamates [91], and oligoribonucleotides containing 2′-*O*-methylribonucleosides [78], 2′-*O*-methylribonucleoside with methylphosphonate linkage [92,93], and 2′-*O*-methylribonucleoside with phosphoramidate linkages (D. Yu, S. Agrawal, unpublished data), and 2′,5′-linked oligoribonucleotides [94].

The positioning of these analogs of oligonucleotides in a given sequence is crucial to the outcome and should be chosen carefully. We have studied two types of MBOs: end-modified and centrally modified MBOs.

10. End-modified MBOs

In end-modified MBOs, a non-RNase-H-activating analog of oligonucleotide is placed at the 3′-end or at both the 3′- and 5′-ends of the PS-oligo (Fig. 2). The purpose of incorporating various modified oligonucleotides in MBOs is to modulate biophysical, biochemical, or biological properties [71–73,78,80–82,86–94]. It has become evident that they also provide improvement in pharmacokinetic and safety profiles. The end-modified MBOs that have been studied extensively contain either methylphosphonate internucleotide linkages [81] or 2′-*O*-methyloligoribonucleosides [78].

End-modified MBOs, in general, have increased in vivo stability due to their increased resistance towards nucleases [78,95]. Because of this feature, some of the end-modified MBOs have shown good bioavailability following oral or colorectal administration [96,97]. The advantage of increased in vivo persistence of MBOs over PS-oligo is that it may allow less frequent administration for pharmacological activity.

Appropriate placement of the modified oligo in an end-modified MBO can decrease the toxicity of PS-oligos [98]. Oligo **5** (Table 1) administered to rats showed a toxicity profile similar to that of other PS-oligos; alteration or modification of the CpG dinucleotides reduced the toxicity (oligos **6**, **7**, and **8**, Table 1 and Fig. 3B). Incorporation of four 2′-*O*-methylribonucleosides at both the 3′- and 5′-ends of oligo **5**, in which the CpG motif was also modified, produced oligo **9**, which showed reduced toxicity (Fig. 3B). Incorporation of four 2′-*O*-methylribonucleosides at both the 3′- and 5′-ends of oligo **5**, in which the CpG motif was not modified, produced an increase in toxicity [60]. An improved safety profile was also observed with an end-modified MBO (oligo **13**) of oligo **10**. (Fig. 2) [97]. The end-modified MBOs in both cases showed similar or improved biological activity. Oligo **13** is presently in Phase I human clinical trials and have shown overall improved safety profile including complement activation, prolongation of aPTT and thrombocytopenia [99].

End-modified MBOs also produce fewer polyanionic-related side effects than do PS-oligos [71,72]. To further minimize the polyanionic characteristics of PS-oligos, attempts have been made to reduce the number of phosphorothioate linkages in MBOs by incorporating 2′-*O*-alkylribonucleosides along with a phosphodiester backbone [79]. Incorporation of 2′-*O*-methyloligoribonucleotides containing phosphodiester linkages at both the 3′- and 5′-ends of the PS-oligo failed to provide nuclease stability comparable to that of the PS-oligo [79]. Similar results have been observed with bulkier 2′-*O*-alkyl groups, including 2′-*O*-propylribonucleosides [58]. Recently, MBOs containing 2′-*O*-methoxyethoxyribonucleosides have been studied, but there is no report of in vivo stability of these MBOs yet [100]. We recently reported that in end-modified MBOs, the number of phosphorothioate linkages can be reduced by introducing alternative phosphodiester and phosphorothioate linkages in a 2′-*O*-methylribonucleoside segment without compromising the nuclease stability [101]. These end-modified MBOs have produced significantly less prolongation of aPTT [101], suggesting that by careful balance, the number of phosphorothioate linkages can be reduced to minimize protein binding without affecting in vivo disposition.

11. Centrally modified MBOs

In centrally modified MBOs, the modified oligonu-

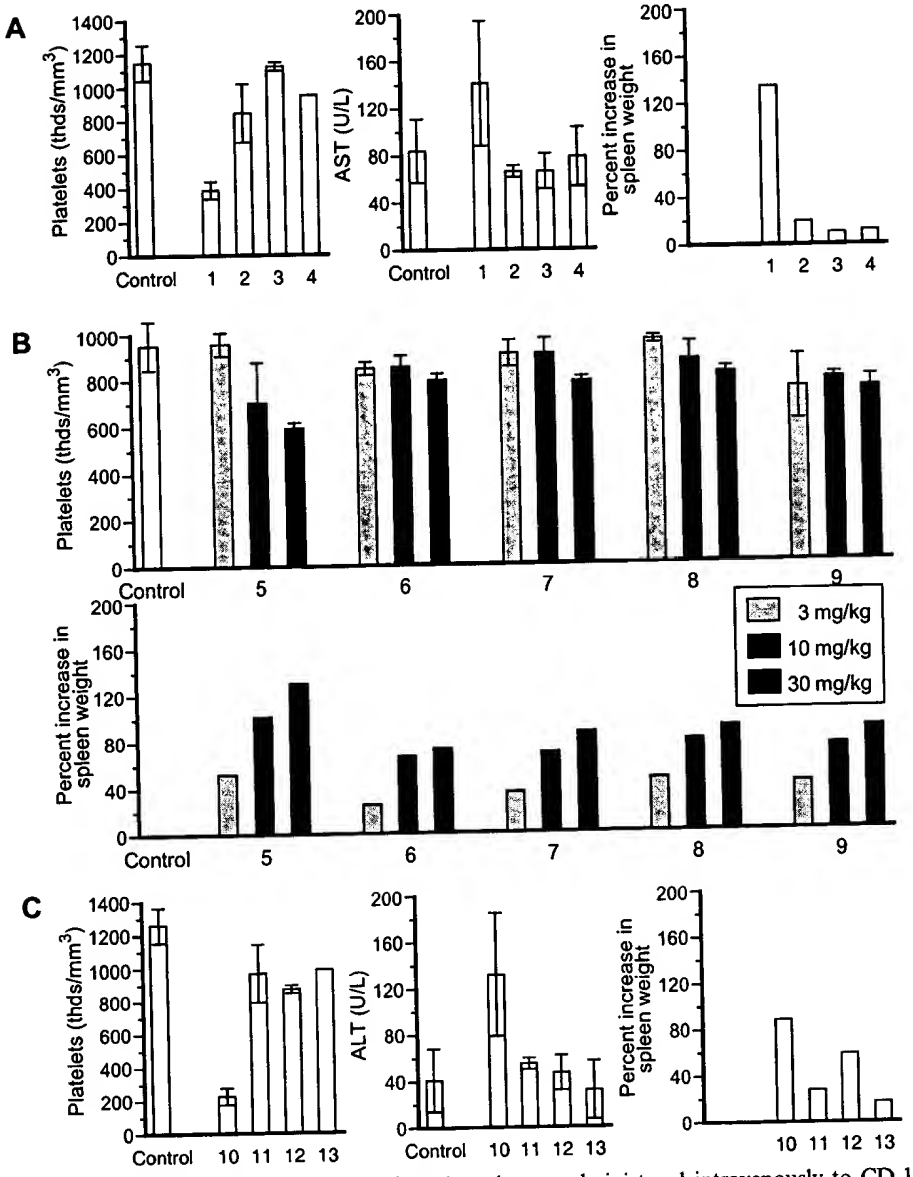

Fig. 3. Toxicity of oligonucleotides in mice and rats. (A) Oligos **1** to **4** were administered intravenously to CD-1 mice at a dose of 15 mg/kg daily for 7 days. On day 8, mice were killed and their spleens removed and weighed. Blood samples were taken for platelet counts and levels of serum aspartate aminotransferase (AST). Oligo **1** caused a decrease in platelet count and an increase in AST levels and spleen weight. Modification of the CpG motif in oligos **2**, **3**, and **4** resulted in minimization of these side effects. (B) Oligos **5** to **9** were administered intravenously to Fischer-344 rats at doses of 3, 10, and 30 mg/kg daily for 7 days. On day 8, the rats were killed and their spleens removed and weighed. Blood samples were taken for platelet counts. Oligo **5** caused a dose-dependent decrease in platelet count and an increase in spleen weight. Modification of the CpG motif is in oligos **6**, **7**, **8**, and **9** had some minimization of these side effects. (C) Oligos **10** to **13** were administered intravenously to CD-1 mice at a dose of 30 mg/kg daily for 7 days. Samples were processed by the same procedure as in the case of A. Oligo **10** caused a decrease in platelet count and an increase in ALT levels and spleen weights compared to oligos **11**, **12**, and **13**, suggesting that flanking sequence and site of CpG motif in PS-oligo is critical for its impact on toxicity. For details of the above protocol, please refer to Agrawal et al. [60].

cleotide is incorporated in the center of the PS-oligo [82,83]. The main advantage of centrally modified MBOs over PS-oligos is that they have few polyanionic-related side effects because they have shorter segments of PS-oligos [82,83]. In addition, oligonucleotides (including 2′-*O*-alkylribonucleotides) containing phosphodiester linkages can be incorporated. Because of the presence of PS-oligos at both the

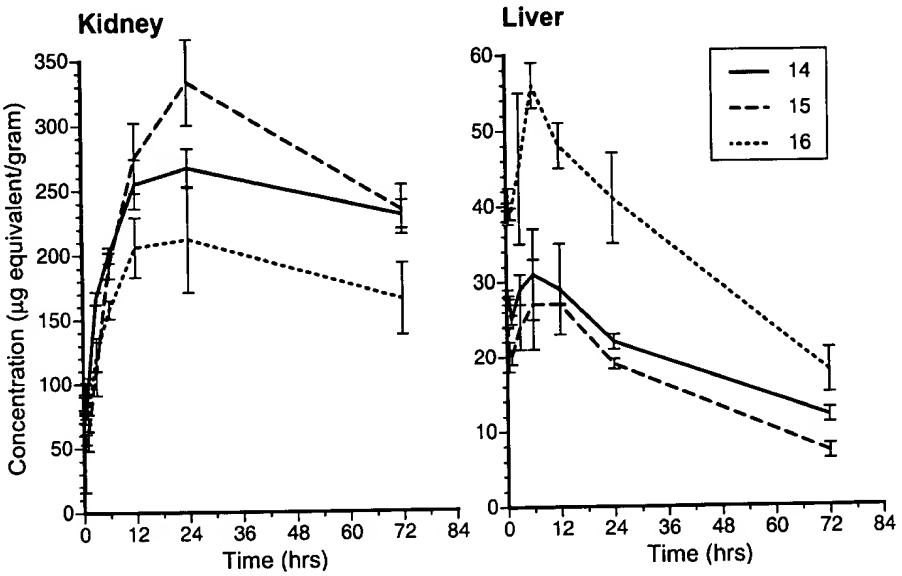

Fig. 4. Impact of number of phosphorothioate linkages on tissue disposition. Oligos **14**, **15**, and **16** were administered to mice at a dose of 30 mg/kg, intravenously. At various time points, animals were killed and various pharmacokinetic parameters were studied (see [83] for details). Disposition of oligo **14**, **15**, and **16** in kidney and liver showed that oligo **14** and **15**, which have reduced phosphorothioate linkages, had increased accumulation in kidney and decreased accumulation in liver compared to oligo **16** which is completely phosphorothioate. Also, increased urinary elimination was observed with oligos **14** and **15** compared to oligo **16** [83].

3′- and 5′-ends, the centrally modified oligonucleotide region is not exposed to exonucleases and therefore remains intact [83]. We have studied centrally modified MBOs containing methylphosphonate [82,83] and 2′-*O*-methylribonucleosides containing phosphorothioate and phosphodiester linkages, and have observed improvements over PS-oligos in many respects [82,83].

In general, MBOs have provided encouraging results over PS-oligos and are being explored for their therapeutic potential in human clinical trials [99]. Other modifications are being explored as well, to further improve the therapeutic potential.

12. Structural modification of PS-oligos

To improve the nuclease stability and minimize the polyanionic nature of oligonucleotides, self-stabilized oligonucleotides have been developed [102,103]. These self-stabilized oligonucleotides are PS-oligos that contain a hairpin structure at the 3′-end. This hairpin structure gets destabilized in the presence of target RNA, thereby allowing the antisense oligonucleotide to bind to target mRNA. Self-stabilized oligonucleotides have been studied for their biophysical, biochemical, and biological activities [102,103], and their pharmacokinetic [104] and safety profiles [61].

13. Bio-reversible analogs of PS-oligos

To improve cellular uptake and minimize undesirable polyanionic-related side effects, we have synthesized and studied PS-oligos that have been derivatized by attaching an acyloxyalkyl group to the internucleotide sulfur moiety; these are referred to as pro-drugs of oligos [105–107]. Incubation of pro-drugs of oligos with esterase bio-reverts the pro-drug to the parent PS-oligo. The rationale behind the use of a pro-drug is that by using the appropriate parent ester group, it would be possible to design pro-drugs for sustained release, site-specific targeting, and oral bioavailability, in addition to above mentioned advantages.

14. Delivery of oligonucleotides

It is important to note that while the sequence of oligonucleotides and its modification are important

factors in antisense activity, delivery of oligonucleotides, including tissue disposition, degradation, and elimination are also important factors for their efficacy in vivo. Oligonucleotides containing phosphodiester internucleotide linkages are rapidly degraded following their administration in vivo [52]. PS-Oligos have increased stability and are widely distributed to major tissues [51,52]. End-modified MBOs containing 2′-*O*-methylribonucleosides and phosphorothioate linkages have shown tissue disposition in animals similar to that observed with PS-oligos, and a significant increase in stability has been achieved [95]. Binding of PS-oligos to serum protein serves as a reservoir, saturation of which results in rapid elimination of PS-oligos in urinary excretion [51]. Aspirin, which affects PS-oligos by binding to serum proteins, has been shown to alter the pharmacokinetics [108]. Reduction in serum protein binding of oligos by introduction of non-ionic linkages (e.g., methylphosphonate) or with increased phosphodiester linkages (e.g., 2′-*O*-methylribonucleotides) has also resulted in increased disposition of administered oligonucleotide to kidney and elimination in urinary excretion [83]. By careful balance of the number of phosphorothioate linkages and other modified oligonucleotides, the rate of elimination and preferential tissue disposition can be achieved [101]. Oligos **14** and **15** (Table 1), which have fewer phosphorothioate linkages, were found less in the liver and more in the kidney than was oligo **16**, which contained all phosphorothioate linkages [83]. Similar results have been obtained with PS-oligos containing 2′-*O*-propylribonucleosides and 2′-*O*-methoxyethoxyribonucleosides containing phosphodiester backbone [58,100] (Fig. 4).

In addition to parenteral route for oligonucleotide administration, we also explored non-parenteral routes, including oral and colorectal [96,97]. PS-Oligos, when administered to mice by gavage, showed good metabolic stability in the stomach; however, extensive degradation was observed in the lower part of the gastrointestinal tract [96,97]. End-modified MBOs containing either 2′-*O*-methylribonucleosides with phosphorothioate linkages or MBOs containing methylphosphonate internucleotide linkages showed improved metabolic stability in the stomach and lower part of the gastrointestinal tract compared to PS-oligos. Tissue disposition studies in mice showed that end-modified MBOs were absorbed when administered by oral gavage and were distributed to major tissues. Similar results of absorption have been obtained following colorectal administration of end-modified MBOs [97].

The bioavailability of a drug is generally calculated based on the concentration of the drug in the plasma compartment versus time (area under the curve (AUC)) [109–111]. Oligonucleotides have short plasma residence times and are rapidly cleared to and retained by the tissues. The half-life of the oligonucleotide in plasma depends on serum protein binding and saturation, which may alter the distribution significantly. Based on our experience with various oligos, we have reached the conclusion that plasma pharmacokinetic parameters do not provide the whole picture in terms of bioavailability in the case of oligonucleotides. Concentration of oligonucleotides in tissues should also be considered when calculating the bioavailability.

15. Future directions

Rapid strides are being made in understanding the rules that govern the effective use of antisense oligonucleotides. It is clear that oligonucleotides can exert biological effects by multiple mechanisms, and the therapeutic potential of oligonucleotides can be explored based on these mechanisms. The sequence of an oligonucleotide is one of the key factors in controlling its mechanism of action and specificity. Selected oligonucleotide sequences can be appropriately modified to enhance the desirable properties and minimize the undesirable properties for their intended uses. Ongoing studies with second-generation oligonucleotide MBOs will further guide us in improving therapeutic potential of antisense oligonucleotides. These modified oligonucleotides which have specific mechanism of action can be used widely for gene target validation as well.

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